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The molecular basis of galactosemia – past, present and future

David J Timson*, School of Biological Sciences, Queen's University Belfast, Medical Biology Centre, 97 Lisburn Road, Belfast, BT9 7BL. UK.

* Corresponding author. School of Biological Sciences, Queen's University Belfast, Medical Biology Centre, 97 Lisburn Road, Belfast, BT9 7BL. UK.

Tel: +44(0)28 9097 5875

Fax: +44(0)28 9097 5877

Email: d.timson@qub.ac.uk

Abstract

Galactosemia, an inborn error of galactose metabolism, was first described in the 1900s by von Ruess. The subsequent 100 years have seen considerable progress in understanding the underlying genetics and biochemistry of this condition. Initial studies concentrated on increasing the understanding of the clinical manifestations of the disease. However, Leloir's discovery of the pathway of galactose catabolism in the 1940s and 1950s enabled other scientists, notably Kalckar, to link the disease to a specific enzymatic step in the pathway. Kalckar's work established that defects in galactose 1-phosphate uridylyltransferase (GALT) were responsible for the majority of cases of galactosemia. However, over the next three decades it became clear that there were two other forms of galactosemia: type II resulting from deficiencies in galactokinase (GALK1) and type III where the affected enzyme is UDP-galactose 4'-epimerase (GALE). From the 1970s, molecular biology approaches were applied to galactosemia. The chromosomal locations and DNA sequences of the three genes were determined. These studies enabled modern biochemical studies. Structures of the proteins have been determined and biochemical studies have shown that enzymatic impairment often results from misfolding and consequent protein instability. Cellular and model organism studies have demonstrated that reduced GALT or GALE activity results in increased oxidative stress. Thus, after a century of progress, it is possible to conceive of improved therapies including drugs to manipulate the pathway to reduce potentially toxic intermediates, antioxidants to reduce the oxidative stress of cells or use of "pharmacological chaperones" to stabilise the affected proteins.

Keywords: Galactose 1-phosphate uridylyltransferase; galactokinase; UDP-galactose 4'-epimerase; Leloir pathway; inherited metabolic disease

Introduction: galactosemia

Galactosemia is a group of three inherited metabolic diseases characterised by the inability to metabolise the aldose monosaccharide galactose (Fridovich-Keil , 2006; Fridovich-Keil & Walter , 2008). This is especially important in young mammals since the main sugar present in milk is lactose, a disaccharide of galactose and glucose. In the most severe forms, the disease manifests as a life-threatening, progressive loss of function of a number of tissues and organs including the ovaries and brain (Waggoner *et al.* , 1990; Schweitzer *et al.* , 1993; Ridel *et al.* , 2005; Rubio-Gozalbo *et al.* , 2010; Fridovich-Keil *et al.* , 2011; Berry , 2012; Waisbren *et al.* , 2012; Karadag *et al.* , 2013; Potter *et al.* , 2013; Timmers *et al.* , 2015). As a consequence it can be associated with significant pathology and cognitive disability in childhood (Bosch , 2006; Timmers *et al.* , 2011). However, these outcomes vary widely and the mildest forms of the diseases are essentially asymptomatic. Currently, the only treatment is the restriction of galactose (and lactose) from the diet (Holton , 1996; Gleason *et al.* , 2000). This treatment is unsatisfactory in many cases, especially in childhood; however, in many countries, it is relaxed in adult patients and this is generally considered to be safe (Van Calcar *et al.* , 2014; Adam *et al.* , 2015). In severe cases of the disease, it tends to slow or reduce the development of symptoms but does not always prevent them (Gitzelmann & Steinmann , 1984; Widhalm *et al.* , 1997).

In the majority of organisms, galactose is mainly metabolised by the Leloir pathway (Figure 1). This short metabolic pathway converts α-D-galactose into glucose 1-phosphate (Frey , 1996). This compound can be isomerised into glucose 6-phosphate by the action of phosphoglucomutase (PGM, EC 5.4.2.2). Thus, galactose is converted into a glycolytic intermediate at the cost of one molecule of ATP per molecule of galactose. Since the first enzyme of the pathway is highly specific for the α-anomer of D-galactose, another enzyme aldose 1-epimerase (GALM; EC 5.1.3.3) catalyses the equilibrium between the α- and β-forms of the sugar (Bailey *et al.* , 1969; Timson & Reece , 2003b;

Thoden *et al.* , 2004). Three types of galactosemia are recognised. The most common, type I or classical galactosemia (OMIM #230400), was the first to be discovered. It has an estimated incidence of approximately 1/30,000; however it is much higher in some groups most notably Irish travellers for whom the frequency is 1/480 (Murphy *et al.* , 1999; J. M. Flanagan *et al.* , 2010; Coss *et al.* , 2013). This disease results from mutations in the gene encoding galactose 1-phosphate uridylyltransferase (GALT; EC 2.7.7.12) (Leslie *et al.* , 1992; Tyfield *et al.* , 1999; T. J. McCorvie & Timson , 2011a; T. J. McCorvie & Timson , 2011b). The range of symptoms is wide ranging from relatively mild to life threatening (Fridovich-Keil & Walter , 2008). In contrast, type II galactosemia (OMIM#230200) is the mildest form of the disease with only early onset cataracts confirmed as a consequence of the disease (Bosch *et al.* , 2002). Dietary restriction of galactose often resolves these cataracts, particularly if the disease is detected early through a screening programme (Hennermann *et al.* , 2011; Janzen *et al.* , 2011). Type II galactosemia is caused by mutations in the gene encoding galactokinase (GALK1; EC 2.7.1.6) (Stambolian *et al.* , 1995; Bergsma *et al.* , 1996; Holden *et al.* , 2004; Timson *et al.* , 2009). Type III galactosemia (OMIM#230250), most likely the rarest and currently the least studied form of the disease, results from mutations in the gene encoding UDP-galactose 4'-epimerase (GALE; EC 5.1.3.2) (Timson , 2006). It is still common to see this disease described as occurring in two forms: a very mild (or "peripheral") form or a severe (or "generalised") form. This concept was decisively debunked almost a decade ago: like the other two types of galactosemia, type III is a continuum disease in which the precise manifestations in each patient are determined by a combination of genotype and environment (Openo *et al.* , 2006).

The discovery of type I galactosemia: from disease to gene

The first recognised report of galactosemia was made by the Austrian ophthalmologist August von Ruess in 1908 (Von Reuss , 1908). However, the first detailed report was made in 1917 by Friedrich Göppert (Göppert , 1917). (As an interesting aside, Friedrich Göppert's scientific achievements have

been largely overshadowed by those of his daughter, the Nobel Prize winning physicist Maria Goeppert-Mayer (Goeppert-Mayer , 1963).) This report of excess galactose in the urine of a patient recognised that the disease had an inherited element. The child concerned had reduced cognitive development and it was observed that feeding him cottage cheese, in which the bulk of the lactose and galactose have been partly metabolised by the bacteria present, reduced the concentration of galactose in the urine (Göppert , 1917; Shahani & Chandan , 1979). In the first half of the twentieth century a number of reports of the disease (sometimes misleadingly called "galactose diabetes") appeared. These established that the disease tends to manifest in early childhood, can be partially reversed by the removal of galactose from the diet and that the liver is one the main organs affected (Mason & Turner , 1935; Bruck & Rapoport , 1945; Mellinkoff *et al.* , 1945; Goldbloom & Brickman , 1946; Greenman & Rathbun , 1948; Bell *et al.* , 1950).

The bulk of these studies were conducted before the metabolic pathway for the catabolism of galactose was fully elucidated. This discovery of this pathway was almost entirely due to the pioneering work of the Argentinian biochemist, Louis Leloir who earned the Nobel Prize in 1970 for this and related work on sugar-nucleotides (Cabib , 1970; L. F. Leloir , 1983). Prior to the elucidation of the pathway, Leloir and others had investigated the phosphorylation of galactose at the expense of ATP in a reaction catalysed by galactokinase (Reiner , 1947; Trucco *et al.* , 1948; Spratt , 1949; Wilkinson , 1949). Leloir's key observation was that galactose is transformed into a glucose derivative, most likely glucose 6-phosphate, in a reaction with required at least one other enzyme and a heat-resistant cofactor (Caputto *et al.* , 1949). This overall conversion required a Walden inversion (i.e. the reversal of stereochemistry at a specific position in the molecule) and the enzyme was tentatively named galactowaldenase. Subsequent work demonstrated that the cofactor was UDP-glucose (Caputto *et al.* , 1950; Cardini *et al.* , 1950). This compound is a representative of a group of sugar derivatives important not only in this pathway, but also in the synthesis of polysaccharides and the oligosaccharide moieties of glycoproteins and glycolipids. Leloir's critical role in their discovery is recognised by these compounds sometimes being referred to as "Leloir

sugars". The transformation of UDP-glucose into UDP-galactose was shown to be part of the overall process (L. F. Leloir , 1951; Paladini & Leloir , 1952). It became clear that the enzyme "galactowaldenase" catalyses two distinct reactions: the epimerisation of the galactose moiety in UDP-galactose and the transfer of the uridyl group onto galactose 1-phosphate (L. F. Leloir , 1951). The interconversion of UDP-galactose and UDP-glucose (the step of the Leloir pathway which alters the sugar stereochemistry) is now known to be catalysed by UDP-galactose 4'epimerase (GALE, Figure 1) and galactowaldenase was increasingly used to refer to this enzyme. GALE was shown to require NAD^+ (then known as diphosphopyridine nucleotide, DPN) as an essential cofactor (Maxwell , 1956). The other aspect of the "galactowaldenase" reaction, the transfer of a uridyl group to galactose 1-phosphate, is catalysed by galactose 1-phosphate uridylyltransferase (GALT, Figure 1).

It was not until 1956 that the genetic nature of the disease was elucidated by Kalckar and co-workers (Isselbacher *et al.* , 1956). The same research group had already demonstrated that galactosemic patients lacked GALT and GALE activity and accumulated the intermediate galactose 1-phosphate (Kalckar Anderson Isselbacher , 1956a; Kalckar Anderson Isselbacher , 1956b). However, GALE activity could be restored in cell extracts by addition of NAD^+ (Isselbacher *et al.* , 1956). On this basis, it was concluded that galactosemia was a single gene disorder resulting from one or more mutations in the gene coding for GALT. This work also provided the basis for definitive tests for the disease – measurement of either galactose 1-phosphate accumulation or lack of GALT activity (Donnell *et al.* , 1963; W. G. Ng *et al.* , 1964). Since GALT activity was reduced in otherwise asymptomatic relatives of patients it was concluded that galactosemia is normally a recessive condition (Hsia *et al.* , 1958; Hugh-Jones *et al.* , 1960).

The human *GALT* gene was assigned to chromosome 3 in 1974, to chromosome 2 in 1975 and to chromosome 9 in 1978 (Tedesco *et al.* , 1974; Chu *et al.* , 1975; Meera Khan *et al.* , 1978; Westerveld *et al.* , 1978; Benn *et al.* , 1979; Mohandas *et al.* , 1979). The GALT genes (*GAL7*) from two yeast species (*Saccharomyces cerevisiae* and *Kluyveromyces lactis*) were among the first to be sequenced,

providing useful information to enable to the search for homologues in other species (Citron & Donelson , 1984; Riley & Dickson , 1984). The coding sequence of the human gene was determined in 1988 (Reichardt & Berg , 1988) and the first disease-associated mutations identified in 1991 (Reichardt & Woo , 1991). Genomic sequencing revealed that human *GALT* is arranged into 11 exons (Leslie *et al.* , 1992). A mutation which changes glutamine 188 to arginine (p.Q188R) was shown to be the most common cause of galactosemia in Caucasians (Reichardt *et al.* , 1991; Leslie *et al.* , 1992). This mutation accounts for 63-90% of cases of type I galactosemia in this ethnic group (Suzuki *et al.* , 2001; Coss *et al.* , 2013). In African populations (and groups descended therefrom) the p.S135L variant is the most common (Lai *et al.* , 1996). The *GALT* gene's location on chromosome 9 was finally confirmed by the human genome project (Lander *et al.* , 2001; Venter *et al.* , 2001). There are now over 200 disease-associated mutations in the *GALT* gene (Calderon *et al.* , 2007; d'Acierno *et al.* , 2009). Of these, the vast majority of these result in single amino acid changes. Two databases of these mutations have been created. One focuses on documenting disease-associated mutations (www.arup.utah.edu/database/galt/galt_welcome.php (Calderon *et al.* , 2007)) and the other on the effects of these mutations on GALT's structure and function (http://bioinformatica.isa.cnr.it/galactosemia-proteins-db/index3.html (d'Acierno *et al.* , 2009; d'Acierno *et al.* , 2014)).

Two more types of galactosemia: types II and III

The belief that all cases of galactosemia resulted from dysfunction of GALT was challenged by the discovery, in 1967, of two children with cataracts and high blood galactose concentrations (Gitzelmann , 1967). Clinical chemistry investigations demonstrated that GALT activity was normal and extracts from blood cells were able to metabolise galactose 1-phosphate. However, galactokinase activity was not detectable (Gitzelmann , 1967). Therefore, galactokinase deficiency must also result in a form of galactosemia. Further cases were reported in the following years, some

being detected through large-scale screening programmes (Thalhammer *et al.* , 1968; Olambiwonnu *et al.* , 1974). Like type I galactosemia, heterozygotes were largely asymptomatic except for reduced blood enzyme activity (Mayes & Guthrie , 1968; Pickering & Howell , 1972).

The galactokinase gene was located to chromosome 17 (S. Elsevier *et al.* , 1974; Orkwiszewski *et al.* , 1974; S. M. Elsevier *et al.* , 1975). Cloning and sequencing of the gene was complicated by the unexpected existence of a second galactokinase-like sequence in the human genome, *GALK2*. This gene encodes the structurally and functionally related protein N-acetylgalactosamine kinase (EC 2.7.1.157), an enzyme which has only minimal activity towards galactose (Lee *et al.* , 1992; Ai *et al.* , 1995; Thoden & Holden , 2005; Agnew & Timson , 2010). The coding sequence for *GALK2* was determined three years before that the *GALK1* was elucidated in 1995 (Stambolian *et al.* , 1995). This study also identified two disease-associated mutations (Stambolian *et al.* , 1995). The genomic sequence of *GALK1* showed that the gene spans eight exons on chromosome 17 (Bergsma *et al.* , 1996). Approximately 40 disease-associated mutations in *GALK1* are now known (Holden *et al.* , 2004; Timson *et al.* , 2009).

In 1981, Holton and coworkers reported the case of a child who had similar symptoms to patients with classical galactosemia, but with normal GALT activity and diminished GALE activity (Holton *et al.* , 1981). It was noted that treatment of this third form of galactosemia by dietary galactose restriction might be particularly problematic. In unaffected individuals, UDP-galactose (a key precursor in glycoprotein and glycolipid synthesis) can be synthesised either from galactose through part of the Leloir pathway or from glucose which is converted to UDP-glucose and then epimerised to UDP-galactose by GALE. In type III galactosemia the second route is not available and a balance needs to be made between restricting galactose intake and providing enough for the synthesis of UDP-galactose (Holton *et al.* , 1981). Interestingly, Holton was not the first to describe a case of reduced GALE activity. Almost a decade before, Gitzelmann described the case of a patient with

reduced GALE activity but no symptoms of galactosemia (Gitzelmann , 1972; Gitzelmann *et al.* , 1977).

Further patients with type III galactosemia were identified and the disease was classified clinically into two forms – an essentially benign peripheral form and a severe, generalised form (Garibaldi *et al.* , 1983; Henderson *et al.* , 1983; Sardharwalla *et al.* , 1988; W. G. Ng *et al.* , 1993; Walter *et al.* , 1999). In the case of the peripheral form, the only manifestation was altered levels of galactose and some metabolites in the blood and no intervention was normally recommended. The division between the two forms was challenged by the identification of a number of mutations associated with an "intermediate" form of the disease (Openo *et al.* , 2006). Of the three types of galactosemia, type III has the smallest number (~25) of identified disease-associated mutations (Timson , 2006; T. J. McCorvie & Timson , 2013; T. J. McCorvie & Timson , 2014). However, these mutations result in a range of severity of symptoms demonstrating that rather than being a binary (or tertiary) condition, type III galactosemia results in a range of outcomes from the almost benign to life-threatening (Openo *et al.* , 2006).

The human *GALE* gene was mapped to chromosome 1 (Benn Shows *et al.* , 1979; Lin *et al.* , 1979). The coding sequence was determined in 1995 (Daude *et al.* , 1995). Genomic DNA sequencing showed that the gene is organised into 11 exons and five mutations associated with type III galactosemia were identified (Maceratesi *et al.* , 1998). The most common mutation associated with a severe form of the disease, which codes for p.V94M, was discovered in 1999 (Wohlers *et al.* , 1999). In addition to its role in the Leloir pathway, human GALE also catalyses the interconversion of N-acetylgalactosamine and N-acetylglucoasamine (Piller *et al.* , 1983; Schulz *et al.* , 2004). This reaction is important in maintaining the pools of UDP-sugars used in the synthesis of glycoproteins and glycolipids and loss of this activity may explain the abnormal glycosylation patterns seen in some cell culture and animal models of type III galactosemia (Kingsley *et al.* , 1986; Rosoff , 1995; Brokate-Llanos *et al.* , 2014).

The present: modern molecular methods applied to galactosemia

The discovery of the coding sequences for *GALT*, *GALK1* and *GALE* opened the door to the application of molecular biology studies. Of particular note, it enabled determination of protein structures, detailed biochemical studies using recombinant proteins and the use of "model organisms" to study the disease. To date, the structure of human GALT has not been reported. The structure of the enzyme from *Escherichia coli* was the first to be determined (Wedekind *et al.* , 1995; Wedekind *et al.* , 1996; Thoden *et al.* , 1997). This structure has been used to develop homology models of the human enzyme and all known disease-associated variants (Marabotti & Facchiano , 2005; Facchiano & Marabotti , 2010; d'Acierno *et al.* , 2014). The structure of human GALK1 was solved in 2005 and that of human GALE in 2000 (Thoden *et al.* , 2000; Thoden Wohlers Fridovich-Keil Holden , 2001a; Thoden *et al.* , 2005). In addition to the wild-type structure of GALE, the disease-associated variant p.V94M has also been solved (Thoden Wohlers Fridovich-Keil Holden , 2001b). This is the only variant associated with any type of galactosemia for which an experimental structure is currently known.

All three of the enzymes have been subjected to detailed biochemical studies. Disease-associated variants of GALT tend to have lower enzymatic activity and some are less able to dimerise when compared to the wild-type (Wells & Fridovich-Keil , 1997; Lai *et al.* , 1999; T. J. McCorvie *et al.* , 2013). Underlying these defects is a failure of the variant enzymes to fold correctly (T. J. McCorvie *et al.* , 2013). Misfolding is often accompanied by aggregation of the disease-associated variants (Coelho *et al.* , 2014). In the case of galactokinase, defects in enzymatic activity approximately correlate with disease severity (Timson & Reece , 2003a; Sangiuolo *et al.* , 2004). To date, no detailed studies on the effects of disease-associated variants on the folding of the enzyme have been completed. The story is similar for GALE: disease-associated variants tend to have lower activity than the wild-type and this reduction in activity is generally greater in variants associated with

severe forms of the disease (Wohlers & Fridovich-Keil , 2000; Timson , 2005). Some disease-associated variants aggregate when expressed in cultured mammalian cells (Bang *et al.* , 2009). The loss of activity often results from a failure to fold correctly and, in some cases, reduced affinity for the catalytically vital NAD^+ cofactor (Quimby *et al.* , 1997; T. J. McCorvie *et al.* , 2012).

Although studies on isolated enzymes have been useful for understanding the fundamental, molecular basis of the disease, it is also necessary to understand the effects on cells, organs and whole organisms. Over the years, the budding yeast *S. cerevisiae* has proved to be a useful model system for studying both type I and type III galactosemia (Wells & Fridovich-Keil , 1996). This organism is well-suited to the task since it does not require galactose to grow and reproduce. Therefore, strains which lack the genes encoding GALT or GALE (or which carry disease-associated mutations) will be unaffected while growing in glucose. However, if the yeast are switched into media in which galactose is the main carbon source then they may exhibit a phenotype depending on the allele(s) present. The human *GALT* and *GALE* genes are able to complement their yeast orthologues (*GAL7* and *GAL10* respectively) (Fridovich-Keil *et al.* , 1995; Quimby *et al.* , 1997). *S. cerevisiae* has been used a variety of studies on the cellular effects of human *GALT* and *GALE* mutations including the effects of various mutations on cellular metabolite concentrations (for examples see (Riehman *et al.* , 2001; Mumma *et al.* , 2008)). Since heterodimers can form in a heterozygous yeast strain expressing both wild-type and variant GALT, the system is also useful for investigating the effects of heterozygosity. For heterodimers of wild-type and either p.Q188R or p.R333W enzymatic activity was reduced to around 14% and 45% respectively of the wild-type homodimer level (J. P. Elsevier & Fridovich-Keil , 1996; J. P. Elsevier *et al.* , 1996). Homodimers of either p.Q188R or p.R333W had essentially no detectable activity under the same assay conditions (J. P. Elsevier *et al.* , 1996). Heterodimers were also less stable to thermal denaturation than wild-type homodimers (J. P. Elsevier & Fridovich-Keil , 1996). These data suggest that these alleles may be partially dominant notwithstanding the observation that in patients' families heterozygotes normally do not present with the disease, and that the degree of dominance varies with the

mutations present (J. P. Elsevier & Fridovich-Keil , 1996). Similar results were seen with GALE heterodimers expressed in yeast cells. While a wild-type/p.V94M heterodimer had approximately 50% activity (compared to a wild-type homodimer), heterodimers of the wild-type with either p.N34S or p.L183P showed less than 50% activity (Quimby *et al.* , 1997; Wohlers *et al.* , 1999). Homodimers of p.V94M or p.L183P had no detectable activity under similar assay conditions, whereas homodimers of p.N34S had approximately 70% of wild-type activity (Quimby *et al.* , 1997; Wohlers *et al.* , 1999). One intriguing result from yeast is that the endogenous GALT and GALE proteins (Gal10p and Gal7p) colocalise in the cytoplasm indicating that the Leloir pathway enzymes may form a complex (or metabolon) (Christacos *et al.* , 2000). When human *GALT* is substituted for *GAL7*, the GALT protein also colocalises with Gal10p suggesting that metabolon formation is conserved from yeast to humans (Christacos *et al.* , 2000). The consequences of this for galactose metabolism *in vivo* or how it has is affected by disease-associated mutations has not yet been investigated.

Despite its many advantages, *S. cerevisiae* is ultimately limited as a model organism for understanding galactosemia since it is unicellular. In recent years a number of important multicellular models have been developed and have been used to generate interesting results. A mouse model for type I galactosemia was generated in 1996. Although the model recapitulated many of the biochemical phenotypes of galactosemia, acute galactose toxicity and consequent pathology were not observed (Leslie *et al.* , 1996; Ning *et al.* , 2000; Ning *et al.* , 2001). The reasons for this were unclear. However, it has been suggested that upregulation of human tumour suppressor gene aplysia ras homolog I (*ARHI*) in response to the metabolic disturbances associated with galactosemia may be implicated (Lai *et al.* , 2008). The ARHI protein causes increased apoptosis and reduced growth; the gene is not present in rodents potentially explaining the lack of effect in this mouse model (Yu *et al.* , 1999; Bao *et al.* , 2002; Fitzgerald & Bateman , 2004). In the last twelve months a second mouse model for GALT deficiency has been reported. In this case, pathology was observed with the majority of *galt*-null pups fed by mothers on a high galactose diet dying before

weaning (Tang *et al.* , 2014). These pups also showed altered ratios of oxidised:reduced glutathione, consistent with increased oxidative stress and adult females showed reduced numbers of ovarian follicles (Tang *et al.* , 2014). The difference in results between these two studies most likely results from the early feeding of pups with high concentrations of galactose. Increased oxidative stress was also observed in a *Drosophila melanogaster* model of type I galactosemia (Kushner *et al.* , 2010; P. P. Jumbo-Lucioni *et al.* , 2013). This model also showed defects in the nervous system with consequent impacts on locomotion of the flies (Ryan *et al.* , 2012; P. Jumbo-Lucioni *et al.* , 2014).

A mouse model for type II galactosemia in which the *galk1* gene was disrupted showed no phenotype. However, when the mice were further modified so that they expressed aldose reductase they developed cataracts (Ai *et al.* , 2000). A key difference between mice and humans is that mice have much lower expression of aldose reductase in the lens cells of the eye. This enzyme catalyses the conversion of galactose to its corresponding sugar alcohol galactitol (dulcitol) and it is this compound which appears to be responsible for the damaging osmotic influx of water into the lens cells (Hayman & Kinoshita , 1965; Dvornik *et al.* , 1973; Ai *et al.* , 2000). Reactive oxygen species are also implicated in the formation of galactosemic cataracts (Mulhern *et al.* , 2006; Mulhern *et al.* , 2007; Abdul Nasir *et al.* , 2014). *D. melanogaster* and *Caenorhabditis elegans* models of type III galactosemia have also been developed (Sanders *et al.* , 2010; Brokate-Llanos *et al.* , 2014). The fruit fly model demonstrated that GALE is essential for development of the organism (Sanders *et al.* , 2010). It also demonstrated that the two physiologically important activities of GALE (epimerisation of UDP-galactose and UDP-N-acetylgalactosamine) were vital and played different roles in development (Daenzer *et al.* , 2012). Developmental defects were also been observed in the *C. elegans* model (Brokate-Llanos *et al.* , 2014).

It is becoming increasingly apparent that disruption of normal glycosylation of proteins and lipids is also a feature of the pathology of type I and type III galactosemia. Defects in the glycosylation of neuronal cells from a galactosemic patient was first noted in the early 1970s (Haberland *et al.* ,

1971). Reduced levels of galactosylation of proteins from cells and serum derived from patients with type I galactosemia has been observed in several studies (Dobbie *et al.* , 1990; Ornstein *et al.* , 1992; Stibler *et al.* , 1997; Charlwood *et al.* , 1998; Coss *et al.* , 2014). It was hypothesised that this is caused by the reduced levels of UDP-galactose often observed in cells derived from galactosemia patients (W. G. Ng *et al.* , 1989). In addition to decreased galactosylation, increased inappropriate incorporation of other monosaccharide moieties such as fucose has been observed (Sturiale *et al.* , 2005). Similarly, glycolipids from galactosemic patients were shown to have reduced levels of galactose and N-acetylgalactosamine compared to healthy patients; this effect was not reversed by a low galactose diet (Petry *et al.* , 1991). In galactosemic patients, N-linked protein glycosylation is associated with increased amounts of mannose and increased numbers of truncated oligosaccharide chains (Y. Liu *et al.* , 2012; Staubach *et al.* , 2012). O-linked glycosylation of proteins is also affected with increased numbers of shorter oligosaccharides (Y. Liu *et al.* , 2012). Recently, it has been suggested that N-glycosylation patterns could be a valuable biomarker for monitoring the severity of the disease and the effectiveness of treatment regimes (Coss *et al.* , 2012; Coss *et al.* , 2014; Knerr *et al.* , 2015).

The future: a realistic chance for therapy?

Recent biochemical work suggests a number of strategies for improved therapy for galactosemia. It is believed that galactose 1-phosphate build-up in types I and III contributes to toxicity. Therefore, blocking the activity of galactokinase which would prevent the accumulation of this compound may be beneficial (Bosch *et al.* , 2002). The availability of a high resolution structure of GALK1 has enabled structure-based drug design and the discovery of some high affinity specific inhibitors of the enzyme (Wierenga *et al.* , 2008; Tang *et al.* , 2010; Odejinmi *et al.* , 2011; Chiappori *et al.* , 2013; Lai *et al.* , 2014; L. Liu *et al.* , 2015). The observation that GALT deficiency is accompanied by increased oxidative stress suggests that antioxidants may be beneficial. A manganese containing porphyrin

compound which mimics the activity of superoxide dismutase has been shown to be effective in the fruit fly model (P. P. Jumbo-Lucioni Ryan *et al.* , 2013). The use of dietary antioxidants has also been suggested (Timson , 2014). Since protein misfolding is likely to be the fundamental cause of most cases of galactosemia, it may be possible to discover molecules which stabilise and promote proper folding of the variant proteins thus increasing enzymatic activity and reducing the tendency to aggregate. Such "pharmacological chaperones" have the potential to restore enzyme activity and alleviate or prevent the bulk of the symptoms (Ringe & Petsko , 2009; Muntau *et al.* , 2014; Brandvold & Morimoto , 2015). This approach has identified compounds which are being used in the successful treatment of cystic fibrosis and transthyretin amyloidoses (Sampson *et al.* , 2011; Bulawa *et al.* , 2012; Hanrahan *et al.* , 2013). Similar approaches are also being developed for a range of other inherited metabolic diseases including Fabry disease, Pompe disease, methylmalonic aciduria, hyperoxaluria, and phenylketonuria (J. J. Flanagan *et al.* , 2009; Pey *et al.* , 2011; Santos-Sierra *et al.* , 2012; Underhaug *et al.* , 2012; Cammisa *et al.* , 2013; Jorge-Finnigan *et al.* , 2013; Mesa-Torres *et al.* , 2013). Discovering pharmacological chaperones for galactosemia will be challenging; however, the existence of good quality experimental structures or models of the three enzymes together with robust assays for their stability will assist the process. Recently, it has been shown that arginine stabilises GALT, including the variant forms p.Q188R and p.K285N supporting the concept that small molecules can enhance the stability and activity of this protein (Coelho *et al.* , 2015). Since misfolded proteins are likely to be targeted for proteosomal degradation, thus further reducing cellular activity, an alternative approach is to inhibit these degradation processes using proteostasis modulators. These can increase the cellular half-lives of misfolded proteins (Vij , 2011). In a mouse model, proteasome inhibitors were able to partially correct cystathionine β-synthase deficiency (Gupta *et al.* , 2013).

Thus after over a century of scientific progress in the understanding of galactosemia, we are finally poised to put this knowledge into practice and develop better treatments for this inherited metabolic disease. It is unlikely that any one of the treatment strategies outlined above will provide

an adequate therapy for all patients with galactosemia. The existence of three types of the disease and the wide range of disease-associated mutations combined with environmental variability, results in considerable diversity of disease phenotypes. There is also a need to treat altered metabolite levels, disturbed glycosylation patterns and the increase in free radical concentrations. Therefore, combinations of treatment approaches and careful monitoring of patients using a variety of biomarkers is likely to be required. In all cases, dietary restriction of galactose will probably continue to be needed. However, the next 100 years should see impressive advances in the treatment of galactosemic patients and there is potential for therapies to be developed which enable near-normal quality of life for these people.

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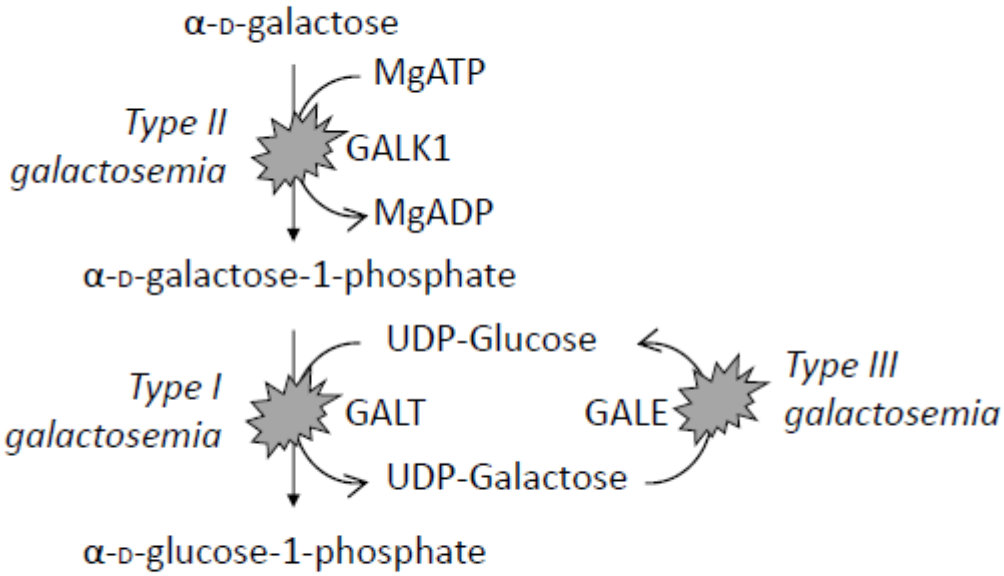
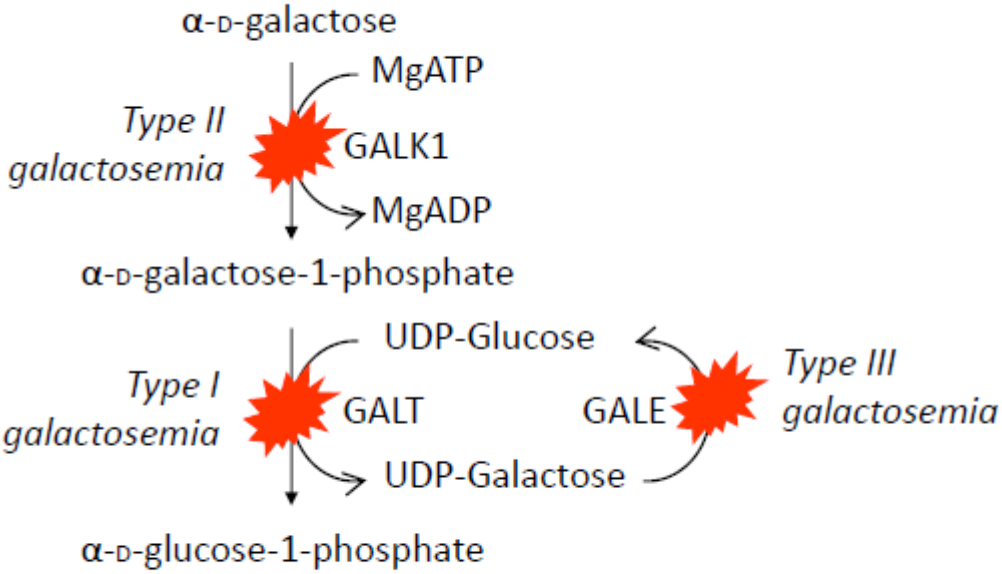
Figure legends

Figure 1: The Leloir pathway of galactose catabolism. In this pathway, α-D-galactose is first phosphorylated at the expense of ATP in a reaction catalysed by galactokinase (GALK1). Impairment of this reaction can result in type II galactosemia. α-D-Galactose 1-phosphate then reacts with UDP-glucose, forming glucose 1-phosphate and UDP-galactose. This reaction is catalysed by galactose 1-phosphate uridylyltransferase (GALT) and deficiency of this enzyme can cause type I galactosemia. The glucose 1-phosphate produced in this reaction can be isomerised to glucose 6-phosphate by phosphoglucomutase and thus enter glycolysis. The reversal of stereochemistry occurs when UDP-galactose is recycled to UDP-glucose in a reaction catalysed by UDP-galactose 4'-epimerase (GALE). Mutations in the gene coding for this enzyme can lead to type III galactosemia. This enzyme also catalyses the interconversion of UDP-*N*-acetylgalactosamine and UDP-*N*-acetyglucosamine. Both reactions are important in maintaining the cellular pools of UDP-sugars for use in the synthesis of glycolipids and glycoproteins.

Figure 1





Graphical abstract

Galactosemia – past, present and future

Abbreviations list

GALK1: Galctokianse

GALK2: N-acetylgalactosamine kinase

GALT: Galactose 1-phosphate uridylyltransferase

GALE: UDP-galactose 4'-epimerase

PGM: Phosphoglucomutase

GALM: Galactose mutarotase

DPN: Diphosphopyridine nucleotide (former name for NAD^+)

The molecular basis of galactosemia: past, present and future

Highlights

- Galactosemia was first described in 1908 by von Ruess
- Leloir discovered the pathway of galactose catabolism
- Kalckar determined that GALT deficiency is the cause of classic galactosemia
- Two other forms exist: GALK1 and GALE deficiency
- Modern molecular approaches are suggesting ways to improve treatment